Signal transduction pathways from exogenous to endogenous salicylic acid in wheat and maize under stress conditions

Main points of the PhD thesis

Orsolya Kinga Gondor

ELTE Doctorate School of Biology (Prof. Dr. Anna Erdei)

Experimental Plant Biology Ph.D program (Prof. Dr. Zoltán Szigeti)

Supervisor:

Dr. Gabriella Szalai

Scientific Consultant

Agricultural Institute, Centre for Agricultural Research,

Hungarian Academy of Sciences

Martonvásár

2016

Introduction

„Stress is „the nonspecific response of the body to any demand madeupon it", that is, the rate at which we live at any one moment." was written byJános Selye in the book „Stress without distress". The salicylic acid (SA) has multiple tasks in plants: It is a phytohormone, a signalmolecule and it can also increase the stresstolerance of the plants. At first the effect of the improved stress resistance was shown in the case of biotic stresses. The mode of action of the SA based on its catalase inhibition. Blocking its activity the level of hydrogene peroxide increases in plants, which could be a signal molecule transmit the effect of SA. In this way it start the acclimation processes which results higher stress tolerance. Recent researches have been shown that the SA has an important role in the abiotic stresstolerance. The increased stress tolerance is really important for the plants, beacuse the plants are stationary so they are not able to escape from the unfavourable environmental aspects, so the only one protection mechanism is to increase of their stress tolerance.

Cadmium is one of the most dangerous heavy metal, because very carcinogenic. Plants accumulate Cd so it cen get into the human body together with the contaminated food. That why this is important to know the mechanism of accumulation and how we could increase the resistance of plants of the cadmium. Wheat seeds were soaked in SA and then they were grown in Cd content hydroponic solution for 56 days. It was measured that the pre-treatment with SA decreased the negative effects of the Cd on the antioxidant system, but caused dramatic changes in the prolin concentration and the membrane-damage too (Agami and Mohamed, 2013).

Aims

It is well known that the SA treatment has protection against different biotic and abiotic stress. During these experiments different plant species and different treatments were used, but there is no information about the mechanism of the different treatments. Our aim is to compare the effect of the different treatments on the plant metabolism and which kind of the protection reactions are induced under stress conditions by the different forms of the SAs. The following tasks were investigated:

- At first we wanted to know is there any kind of difference between the SA seeds soaking or the hydroponic treatment.

- If yes, which physiological parameters change during the treatments and how the different SA biosynthesis pathways and the synthesis of the intermediates can be effected by the different SA treatments.
- Furthermore, how the flavonoid biosynthesis has been influenced by these treatments, because these antioxidant compounds are also synthetised on the phenylpropanoid pathways.
- The next step was, whether the acid and salt (sodium-salicylate; NaSA) of SA provided the same protection for the plants under Cd stress?
- Is there any kind of the physiology parameters, which can describe the condition of the plant, and the antioxidant enzymes activity?
- The heavy metals induce the bioshynthesis of the phytochelatins, that is why we observed the effect of the different SA forms on the bioshynthesis of the phytochelatins.

Materials and methods

Winter wheat plants (*Triticum aestivum* L. var. Mv Emese) were used in the first part of the experiments. Seeds were soaked overnight either in distilled water (control plants) or in 0.5 mM SA (SA seed-soaked; SA-ss plants). The seeds were then germinated for 3 days at 22°C, after which the seedlings were grown in modified Hoagland solution (Pál et al., 2005) for 2 weeks at 20/18°C with 16/8-h light/dark periodicity and photosynthetic photon flux density (PPFD) of 250 μmol m^{-2} s^{-1}in a Conviron G-48 plant growth chamber (Controlled Environments Ltd, Winnipeg, Canada) in the phytotron of the Agricultural Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Martonvásár, Hungary. At the end of this period 0.5 mM SA was added to the hydroponic solution of some of the control plants for one day, after which the solution was replaced with control solution (SA-h plants). Leaf and root samples were collected one and seven days after the one-day hydroponic SA treatment.

For the another experiment maize (*Zea mays* L. Norma hibrid) plants were used. Sterilized seeds of maize (*Zea mays* L., hybrid Norma) were germinated for 3 days at 26 °C, after which six seedlings per beaker were grown in 400 ml modified Hoagland solution (Pál et al., 2005) at 22/20 °C with 16/8-h light/dark periodicity in a Conviron PGR-15 plant growth chamber in the phytotron of the Agricultural Institute. The photosynthetic photon flux density was 240 μmol m^{-2} s^{-1}, provided by metal halide lamps, with a relative humidity of 75 %. After

10 days various treatments were applied. One group of seedlings was treated with 0.5 mM SA or NaSA for 1 day (designated as SA pre, NaSA pre, respectively), after which half of the plants were moved on the original growth solution and the second half were treated with 0.5 mM Cd for 1 day.

Another group of seedlings was treated with 0.5 mM Cd for 1 day without pre-treatment with SA or NaSA, while a third group was treated simultaneously with Cd and either SA or NaSA.

The quantum yield of Photosystem 2 (PS 2), indicated by the ΔF/Fm' [(Fm'- Fs)/Fm' chlorophyll fluorescence induction parameter, where Fm' and Fs represent the maximum and steady-state chlorophyll fluorescence levels in the light-adapted state, respectively, was measured on fully expanded leaves using a pulse amplitude modulated fluorometer (PAM-2000, Walz, Effeltrich, Germany)

In the second experiment chlorophyll-a fluorescence quenching analysis was carried out using a pulse amplitude modulated fluorometer (Imaging-PAM M-Series fluorometer; Walz, Effeltrich, Germany). The Fv/Fm parameter was determined on plants previously dark-adapted for 20 minutes, using a 0.8 s saturating pulse (PPFD=3000 μmol m^{-2} s^{-1}) provided by a LED-Array Illumination Unit IMAG-MAX/L (λ=450 nm). Photosynthesis was then activated using 230 μmol m^{-2} s^{-1} actinic light intensity for 15 min and quenching analysis was performed using 0.8 s saturating pulses applied every 30 s. The quenching parameters were determined under steady state conditions according to the nomenclature described by Klughammer and Schreiber (2008).

For the ion leakage measurement frozen leaf samples were shaken in distilled water, after which the electrolyte leakage was measured on the basis of conductance using an Automatic Seed Analyzer (ASA610, Agro Science).

The root viablity of wheat plants was measured using nitroblue tetrazolium chloride solution according to Gondor et al. (2013) with UV-160A spectrophotometer (Shimadzu, Japan).

The root viability of maize plants was measured after incubation in 2,3,5-triphenyl-tetrazolium chloride solution in the dark for 1 day. The concentration of formed extracted red component was determined using an UV-160A spectrophotometer (Shimadzu, Japan) as written by Gondor et al. (2013) previously.

The lipid peroxidation analysis was based on the measurement of malondialdehyde (MDA) level. After grinding 0.2 g of tissue in 600 μl 0.1% (w/v) trichloroacetic acid, followed by centrifugation at 12 000g for 10 min, 300 μl of the supernatant was mixed with 2 ml of 0.5%

(w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid and incubated at 90°C for 30 min. The MDA concentration was measured spectrophotometrically at 532 nm, with the subtraction of non-specific absorption at 600 nm. The concentration of lipid peroxides, together with the oxidatively modified proteins, was then quantified in terms of the MDA level using an extinction coefficient of 155 mM^{-1} cm^{-1}, and expressed as nM g^{-1} fresh weight (Thomas *et al.*, 2004).

The chlorofill content of the maize was measured before sample collection on the 2nd and 3rd leaves using SPAD-502 chlorophyll meter (Minolta Camera Co., Ltd, Tokyo, Japan).

The antioxidant enzymes were isolated according to Janda et al. (1999) and measured using a Shimadzu UV-VIS 160A spectrophotometer. The glutathione reductase (GR) activity was recorded by measuring the reduction of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 412 nm, as described by Smith et al. (1988). The activity of ascorbate peroxidase (APX) was determined by monitoring the consumption of AA at 290 nm, while catalase (CAT) activity was recorded at 240 nm as the decrease in the hydrogen peroxide quantity (Janda et al. 1999). In the case of guaiacol peroxidase (GPX), activity was detected spectrophotometrically as the increase in absorbance caused by the oxidation of guaiacol (Ádám et al. 1995).

The activity of γ-glutamylcysteine synthetase enzyme was measured from root and leaf samples by method of Hell and Bergmann (1990). The initial and produced amount of γ-glutamylcysteine was measured with HPLC equipments.

The activity of glutathione synthetase was measured from root and leaf samples by method of Hell and Bergmann (1988). The enzyme activity were calculated from the difference between the initial and the produced glutathione amount.

The reduced and oxidized thiol content was determined from leaf and root samples based on the method of Kocsy et al. (2004) with HPLC and fluorescence detector.

The phytochelatins and the activity of phytochelatin synthase determination was based on Chen et al, (1997) with HPLC with UV/VIS detector.

The HPLC analysis of salicylic acid was performed using the method reported by Meuwly and Métraux (1993) after extraction with methanol. Ortho-anisinic acid was used as an internal standard and para-hydroxybenzoic acid as the extraction carrier. The measurements were performed on a Waters HPLC instrument.

The content of cadmium was measured by ICAP-61E type atomic absorption spectroscopy instrument at the HAS CAR Institute for Soil Sciences and Agricultural Chemistry based on Hegedűs et al. (2001).

For the measurement of ascorbate (AA) the samples were homogenised in metaphosphoric acid, and the total AA content was determined by means of dithiothreitol reduction, followed by measurement using an isocratic Waters 2690 HPLC instrument. The quantity of the oxidised form was obtained as the difference between the total and reduced forms.

Samples for ACC and MACC measurements were prepared as described by Tari and Nagy (1994), with some modifications, and ACC was measured by gas chromatography after conversion to ethylene, using the method of Lizada and Yang (1979). The MACC content was calculated as the difference in the ACC content before and after hydrolysis.

Results

- The quantity of MDA increased only in the leaves of SAh wheat plants. The decline in the quantum efficiency of PSII was not drastic, suggesting that the treatment did not cause serious damage to the plants. On the first day after the treatment the quantity of SA increased in all the fractions recorded, but decreased 7 days later. The *o*HCA quantity also rose after the treatment, and was even higher in the free fraction after 7 days. Although the quantity of BA increased after the treatment, it declined again after 7 days.
- The SAss plants exhibited no stress symptoms. In the course of the treatment, components involved in SA biosynthesis showed no change compared with the control.
- CA also increased after the SAh treatment but, since CA is also a precursor for flavonoid biosynthesis, this increase was very slight. The quantity of kaempferol decreased after the SAh treatment, while that of quercetin rose in the free fraction in the leaves. The quantity of rutin, which is synthesised from quercetin, also increased.
- Cd content measurements of plants showed that the pretreatement with SA/NaSA or addition together with Cd also decreased the Cd uptake of the roots. The SA and Cd together addition decreased in the highest manner but the NaSA and Cd added together also affected the uptake. However, we measured high phytochelatin (PC) content after the Cd treatment in the roots, but the highest PC accumulation was measured after the NaSA treatment in roots. It was the same in the leaves as detected in the roots. The high PC accumulation suggests that the PC was synthetised in the roots after the treatment and the plant transported it to the leaves in xylem, because the activity of the phytochelatin sythase (PCS) enzyme did not increase compered to the control. In case of the NaSA treatment the highest the PC content was found in the roots, while neither the Cd

treatment, nor the SA treatment did not increase it so much extent. However the activity of the PCS was the same a sin the control plants after the NaSA treatment. The highest enzyme activity was measured after the SA pretreatment followed by Cd addition in the roots, while the PC content was low. The highest PC accumulation in the leaves was detected after the SA pretreatment followed by Cd addition and when the SA and Cd was added together, but the activity of the PCS was not dramatically.

- On the basis of both photosynthesis parameters and GSH redox potential calculations, plants pretreated with NaSA were in a healthier state than the control plants, indicating that NaSA treatment improved the stress tolerance of the plants. When Cd was applied after SA pretreatment, phytochelatin was synthesised in the roots and then transported into the leaves, while the joint application of SA and Cd resulted in phytochelatin being synthesised in the leaves.

Conclusion

I. The SA is sythetized through the fenilpropanoid pathway from CA via *o*HCA or BA. Based on our measurements these two precursor components content amount was increased measurably 1 day after the treatment, so after the hidroponic treatments the plants activate the fenilpropanoid pathway and inducate the SA production, but 7 days after the treatment was needness so repeled the SA production.

II. The flavonoids are antioxidant compounds which could help to parry the oxidative stress to the plants. The plants induced the antioxidant compounds through the fenilpropanoid pathway.

III. The SA seed-soaked plants did not shown stress syndrome. The temporary activity decrease of CAT was measured only the SA treated plants. Till some of the antioxidant enzyme activity (POD, GR, GST) was decreased while the activity of APX was high the Sah treated plants.

IV. The Cd taken up by untreated maize plants accumulated in the roots, while preliminary treatment with SA resulted in the transport of adsorbed Cd from the roots into the leaves. So the activity of PCS was high after SA pretreatment added Cd in root and the PC was transported to the leaf. The PC was high after the SA and Cd addition together in leaf but the activity of the PCS was not increased, so the PC was sythetased in leaf.

The affect of the SA and NaSA could be different on the antioxidant system. The affect of the SA and NaSA could be also different on the heavy metal transport. The SA mainly facilitate the Cd transport to leaf, while the NaSA increase the PC content in root. To summarize, we examined the different SA forms, acid and salt forms different defence effect under Cd stress. These different are also point out that correct way to interpreted to investigate the effect of SA plants, if we pay attention to the form of SA, because as you can see, according the different mechanism, if other forms used of SA.

References:

Ádám A, Bestwick CS, *et al*, 1995, Planta 197: 240–249.
Agami RA, Mohamed FG, 2013, Ecotoxicol. Environ. Safety, 94:164-171.
Chen J, Zhou J, Goldsbrough PB, 1997, Physiol. Plant., 101: 165-172.
Gondor OK, Janda T, Szalai G, 2013, Acta Agronomica Hungarica 61:(3) 219-226.
Hegedüs A, Erdei S, Horváth G, 2001, Plant Sci. 160(6): 1085-1093.
Hell R, Bergmann L, 1988, Physiol. Plant. **72,** 70-76
Hell R, Bergmann L, 1990, Planta 180, 603-612
Janda T, Szalai G, *et al*, 1999, Planta 208: 175–180.
Kocsy G, Szalai G, *et al*, 2004, Plant Sci., 166: 451-458.
Klughammer C, Schreiber U, 2008, PAM Application Notes 1: 11-14
Lizada MCC, Yang SF, 1979, Anal. Biochem.100, 140-145.
Meuwly P, Métraux JP, 1993, Anal Biochem 214: 500–505.
Pál M, Horváth E, *et al*, 2005, Physiol. Plant 125,356–364.
Smith IK, Vierheller TL, *et al*, 1988, Anal. Biochem 175: 408–413.
Tari I, Nagy M, 1994, Physiol. Plantarum, 90(2): 353-354.
Thomas, J.C., *et al*, 2004, Plant Sci., 167: 259-266.

List of papers forming the basis of the thesis

A Papers published in peer-reviewed journals

Gondor, O.K., Pál, M., Darkó, É., Janda, T., Szalai, G. (2016a): Salicylic acid and sodium salicylate alleviate cadmium toxicity to different extents in maize (*Zea mays* L.). PLoSONE 11(8): e0160157. doi:10.1371/journal.pone.0160157 **IF: 3,54**

Gondor, O.K., Janda, T., Soós, V., Pál, M., Majláth, I., Adak, M.K., Balázs, E., Szalai, G. (2016b): Salicylic Acid Induction of Flavonoid Biosynthesis Pathways in Wheat Varies by Treatment. Front. Plant Sci. 7:1447. doi: 10.3389/fpls.2016.01447 **IF: 4,495**

Janda, T., **Gondor, O.K.,** Yordanova, R., Szalai, G., Pál, M. (2014): Salicylic acid and photosynthesis: signalling and effects. Acta Physiologiae Plantarum 36:(10) pp. 2537-2546. **IF: 1,524**

Gondor, O.K.,, Janda T. and Szalai G. (2013): Comparative study of viability measurement methods in crop plants, Acta Agronomica Hungarica 61:(3) pp. 219-226.

Abstracts in conference proceedings

Our Future, Pannonian Plant Biotechnology conference for PhD students in plant biology: ***Gondor O. K,*** Janda T. and Szalai G: Effects of exogenous salicylic acid on the endogen salicylic acid level and its precursors (*oral*) 2013. p. 34-35. (ISBN: 978- 963- 89129- 2-3)

XIX. Növénynemesítési Tudományos Napok 2013: ***Gondor O. K***., Janda T., Szalai G.: Az exogén és endogén szalicilsav közötti jelátviteli út tanulmányozása búzában és modellnövényekben stresszkörülmények között (*Poster*) 2013. p. 91. (ISBN: 978- 963-9639- 50-8)

Plants for the future, Plant biotechnology for the future of agriculture in the Central European region conference: ***Gondor O. K***., Janda T., Szalai G: Modification of the phenylpropanoid pathway after different salicylic acid treatments in wheat (*Poster*) 2013. p. 17-18

The Plant Biology Europe FESPB/EPSO Congress 2014: ***Gondor O. K***., Janda T., Szalai G: Modification of the phenylpropanoid pathway after different salicylic acid treatments in wheat (*Poster*)

The Plant Biology Europe FESPB/EPSO Congress 2014: ***Gondor O.K.***, Pál, M., Janda, T., Szalai G.,: Protective Effect of Different Forms of Salicylic Acid against Cd Stress in Young Maize Plants. (*Poster*)

A Papers are not related to the thesis but published in peer-reviewed journals

Gondor O.K., Szalai G., Kovács V., Janda T., Pál M.: Impact of UV-B on drought- or cadmium-induced changes in the fatty acid composition of membrane lipid fractions in wheat. (2014) Ecotoxicology and Environmental Safety, 108:129-34. **IF: 2.482**

Gondor O.K., Szalai G., Kovács V., Janda T., Pál M.: Relationship between Polyamines and Other Cold-induced Response Mechanisms in Different Cereal Species. (2016) J. Agronomy and Crop Science 202:(3) pp. 217-230. **IF: 2.565**

Kovács V., **Gondor O.K.,** Szalai G., Darkó É., Majláth I., Janda T., Pál M.: Synthesis and role of salicylic acid in wheat varieties with different levels of cadmium tolerance (2014) Journal of Hazardous Materials 280: pp. 12-19. **IF: 4.331**

V. Kovács; ***O. K Gondor***; G. Szalai; I. Majláth; T. Janda M. Pál: UV-B radiation modifies the acclimation processes to drought or cadmium in wheat (2014) Environmental and Experimental Botany, 100: pp. 122-131. **IF: 3.003**

M. Pál, G. Szalai, V. Kovács, ***O. K. Gondor***, T.Janda: Salicylic Acid-Mediated Abiotic Stress Tolerance (2013) In: Shamsul Hayat, Aqil Ahmad, Mohammed Nasser Alyemeni (eds.) Salicylic Acid Plant Growth and Development. Netherlands: Springer Dordrecht Heidelberg, 2013. pp. 183-247. (ISBN: 978-94-007-6427-9)

M. Pál, ***O. K. Gondor***, T. Janda: Role of salicylic acid in acclimation to low temperature. (2013) Acta Agronomica Hungarica 61:(2) pp. 161-172.

Abstracts in conference proceedings not related to the thesis

Fiatal Biotechnológusok Országos Konferenciája "FIBOK 2014": ***Gondor O. K***., Szalai G., Janda T., Kovács V., Pál M.: Impact of UV-B on drought or cadmium induced changes in fatty acid composition of membrane lipid fractions in wheat. (*Poster*)

Advances in Plant Breeding & Biotechnology Techniques: Book of Abstracts: **Gondor O.K.**, Pál M, Janda T, Szalai G.:Effect of the hardening under different light conditions on maize chilling tolerance.. 96 p.